Assessment of disease activity in Graves' ophthalmopathy
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Association of thyrotropin receptor antibodies with activity rather than severity of Graves’ ophthalmopathy

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Abstract
Graves’ ophthalmopathy (GO) and Graves’ hyperthyroidism are closely associated diseases and thought to be caused by the same autoimmune process. An obvious explanation for this would be the presence of autoantibodies reacting with an autoantigen present in the orbit and the thyroid gland. The TSH-Receptor (TSH-R) is a likely candidate, because it appears to be present in both organs. If TSH-R antibodies are responsible also for the ophthalmopathy one would expect that their titers correlate with clinical characteristics of the eye disease. The aim of the present study is to see whether TSH-R antibodies are related to the activity and severity of the thyroid-associated ophthalmopathy.

TSH-R antibody levels were measured as TBII (TRAK assay), and TSI (cAMP response of TSH-R transfected cell line) in 63 patients with untreated moderately severe GO, accompanying Graves’ thyroid disease; all patients had been euthyroid for >2 months. TBII and TSI titers were strongly related to each other. TBII or TSI titers did not correlate with thyroidal or orbital disease duration, nor TPO antibody levels. In contrast, we found a striking and highly significant correlation between the Clinical Activity Score (CAS) of the eye disease, and both TBII (r= 0.54; P< 0.0001) and TSI (r= 0.50; P< 0.0001). In addition, a weaker but significant relation was found between proptosis (in mm) and TBII (r=0.36; P=0.004) and TSI (r=0.49; P= 0.0001). No correlation was found with eye muscle motility.

In conclusion, TSH-R antibody levels correlate directly with the activity of GO. The results support the hypothesis of a pathogenetic role of TSH-R antibodies and the TSH-R in the orbit in GO.

Introduction
Graves’ hyperthyroidism is an autoimmune disease caused by TSH-Receptor Stimulating Immunoglobulins (TSI), and frequently associated with Graves’ ophthalmopathy (GO). The reason for this association is incompletely understood, but it is likely that the thyroid disease and the ophthalmopathy are manifestations
of one disease entity: Graves' disease. An attractive hypothesis explaining these different manifestations would be the existence of an autoantigen present in both the thyroid gland and the orbit. Autoantibodies (or autoreactive T-cells) against this antigen might then crossreact with both tissues. An obvious candidate for this shared antigen is the TSH-Receptor (TSH-R).

There is accumulating evidence that the TSH-R is indeed present in the orbit,\textsuperscript{1,2} and expressed on orbital fibroblasts.\textsuperscript{3,4} If the orbital TSH-R is responsible for the ophthalmopathy one would expect that TSH-R autoantibodies titers correlate to some degree with the clinical expression of this thyroid-associated ophthalmopathy. However, many authors did not find a relationship between TSH-R antibody levels and the severity of GO.\textsuperscript{5-9} These studies can be criticized for various reasons. First, most studies used a rather crude overall score to assess disease severity, the Ophthalmpathy Index.\textsuperscript{5,8,9} Secondly, the applied TSH-R antibody assays (like the LATS bioassay)\textsuperscript{7} were not very sensitive. Thirdly, most studies included patients with burnt-out inactive GO who might still have rather severe eye disease although the autoimmune attack has ceased. In this situation it is difficult to assess appropriately a putative role for TSI. Still, some studies did find a correlation between Long Acting Thyroid Stimulatory Activity and severity of GO.\textsuperscript{10-12}

Therefore, we hypothesized that if TSH-R antibodies are involved in the pathogenesis of GO their titers might correlate with disease activity, rather than with disease severity. We therefore measured TSH Binding Inhibiting Immunoglobulins (TBI) and TSH-Receptor Stimulating Immunoglobulins (TSI) in 63 patients with GO, and related these titers with the Clinical Activity Score (CAS) of the eye disease.\textsuperscript{13}
Patients and methods

Patients

Sera were drawn from 63 newly referred patients with Graves' ophthalmopathy. They had varying degrees of disease activity, but all had moderately severe GO (defined as proptosis ≥ 25 mm, and/or evident restriction of eye muscle motility). No patients had been treated for their eye disease, but all were euthyroid for > 2 months (defined as normal values of fT4 and T3 in the absence of elevated TSH values).

The activity of the eye disease was assessed by an ophthalmologist, who was unaware of the laboratory data, using the Clinical Activity Score (CAS). This score is based on the classical signs of inflammation: orbital pain, (2 items: spontaneous or on attempted up, side or down gaze), redness of the conjunctiva or eyelids (2), swelling of caruncle, eyelids or chemosis (3), and impaired function (worsening in the last 3 months in proptosis, eye muscle motility, visual acuity (3). The score thus ranges from 0 to 10. The overall severity of the disease was assessed using the Total Eye Score (TES) based on the NOSPECS classification, which was however adapted by using more objective and quantitative data. Soft tissue involvement was graded (0-3) using color slides, and eye muscle involvement was graded (0-3) on the basis of a quantitative measurement of elevation. The TES is calculated as the sum of each class present multiplied by the grade in that class (maximum: 45). Class 5 (corneal involvement) was not included in the TES, in view of its rapid reversibility by the use of artificial tear drops.

Methods

TSH Binding Inhibiting Immunoglobulins (TBII).

TBII were measured using a commercially available radioreceptor assay (TRAK-assay, BRAHMS Diagnostica Berlin GmBH, Germany). All serum samples were assayed in duplicate.
Chapter 4

TSH-Receptor Stimulating Immunoglobulins (TSI).

For this assay we used CHO cells stably transfected with the human TSH-Receptor (clone JP26, a kind gift of Prof Dr. G. Vassart, Brussels). The cells were maintained under 5% carbondioxide at 37°C in RPMI/10%FCS/pen/strep (Biowhittaker, Belgium) with a fortnightly treatment with G418 to maintain selective pressure. The IgG fraction was purified from patients sera using protein A-Sepharose (Pharmacia, Sweden). The final concentration of the IgG fraction after purification was between 4 and 8 mg/ml. Before the experiment, cells were seeded at a density of 4.10^4 cells/well in a 96-well tissue culture dish (Corning/Costar, The Netherlands) in RPMI/10% FCS. After 24 hours the cells were washed and then 100 μl of hypotonic Hanks Balanced Salt Solution (HBSS)/0.5 mM isobutylmethyloxanthine was added with or without 10 μl purified patient IgG and incubated for 2 hours. After 2 hours incubation medium was collected and cAMP production was measured using a commercially available radioimmunoassay (Immunotech, Marseille, France). cAMP data are expressed as total amount of cAMP in pM/well. All samples were assayed in triplicate and in order to minimize the known variability of this bio-assay, all samples were measured in one run. The coefficient of variation of the whole assay procedure was 13±6% per triplicate sample.

Other measurements.

FT4 was measured by a two-step FIA assay (DELFIA, EG&G Wallac, Turku, Finland), TSH by IFMA (DELFIA, EG&G Wallac, Turku, Finland). Total T3 measurements were measured by in house radioimmunoassay. TPO antibodies were measured by immunofluorescence (CLB, Amsterdam, the Netherlands).

Analysis

TSI and TBII measurements were correlated with demographic patient variables, and with disease severity (grade of soft tissue involvement, proptosis in mm, eye muscle motility in degrees of elevation and TES), and disease activity (CAS) by
Spearman’s correlation using SPSS software. If TBII were undetectable (< 5 U/l) a figure of 2,5 U/l was used in the analysis. If cAMP production was > 130 pM/well, 130 pM/well was used in the analysis. In addition, stepwise multivariate regression analysis was done with patients’ age, sex, the duration of GO, and the CAS as independent variables, and TSI as the dependent variable. Comparisons of groups were done using unpaired, two-sided t-tests, or Mann Whitney U tests.

**Results**

The clinical data of the 63 patients are shown in Table 1. TBII were detected in 36/63 (57%) of the patients. TBII and TSI were related to each other (Spearman’s correlation coefficient \( r = 0.67 \), \( p< 0.0001 \); figure 1). TSI (and not TBII) correlated significantly negative with age, however, using stepwise regression analysis age, sex, duration of GO and the CAS indicated only a strongly significant relation with the CAS. TBII or TSI levels were unrelated to duration of Graves thyroid disease or TPO antibodies. There was no correlation between TBII or TSI and the overall severity of the ophthalmopathy, using the total eye score (Table 2). Only in the 27 patients with a short duration of the eye disease (<12 months), the TES did correlate with TBII (\( r= 0.41; \ p= 0.035 \)) and TSI (\( r= 0.43; \ p= 0.027 \)). Assessing disease severity by separate parameters, we found that the patients with more severe soft tissue involvement (NO SPECS 2 b-c) had higher levels of TBII and TSI than the patients with lesser degrees of soft tissue involvement (NO SPECS class 2 0-a) (Table 2). In addition a significant correlation was observed between proptosis and TBII or TSI in all 63 patients (\( r= 0.49\), \( p < 0.0001 \), for the TSI \( r=0.36, \ p=0.004 \) respectively).

There was a consistent and striking correlation between both TBII and TSI levels and disease activity as assessed with the CAS (Figure 2). All patients were (at least > 2 months) euthyroid, at the time of TBII/TSI measurements: TSH (mean ± sd) 1,96 ± 2,20 mU/l, FT4index (mean ± sd) 122 ± 30, T3 (mean ± sd) 1,94 ± 0,51 nmol/l.
Table 1. General characteristics of 63 patients with moderately severe Graves' ophthalmopathy related to the levels of TBII and TSI.

<table>
<thead>
<tr>
<th></th>
<th>TBII Median (range)</th>
<th>r</th>
<th>p value</th>
<th>TSI Median (range)</th>
<th>r</th>
<th>p value</th>
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<tr>
<td>age (years) (mean±sd)</td>
<td>53.0±9.5</td>
<td>-0.109</td>
<td>.43</td>
<td>39.0 (21-30)</td>
<td>-0.33</td>
<td>.008</td>
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<td>duration GTD (months, median and range)</td>
<td>21 (0-312)</td>
<td>0.11</td>
<td>.40</td>
<td>35.0 (23-86)</td>
<td>0.09</td>
<td>.50</td>
</tr>
<tr>
<td>sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>females</td>
<td>n=43</td>
<td>13.0 (2.5-400)</td>
<td>]</td>
<td>39.0 (21-30)</td>
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<td>]</td>
<td>35.0 (23-86)</td>
<td>35</td>
<td></td>
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</tr>
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<td>12   (2.5-274)</td>
<td>]</td>
<td>39 (21-130)</td>
<td>39</td>
<td>.84</td>
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<td>39 (23-130)</td>
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<td></td>
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<td>Thyroid disease</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>antithyroid drugs + T4</td>
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<td>39 (23-130)</td>
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<td>.42</td>
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<td>T4</td>
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<td>]</td>
<td>36.0 (21-130)</td>
<td>36</td>
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</tr>
<tr>
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<td>34 (29-39)</td>
<td>34</td>
<td></td>
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<td>anti-TPO antibodies</td>
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<td>39 (26-130)</td>
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<td>.61</td>
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<td>positive</td>
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<td>]</td>
<td>37.5 (21-130)</td>
<td>37</td>
<td></td>
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<tr>
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<td>n=9</td>
<td></td>
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</table>

TBII receptor binding inhibiting immunoglobulins (TBII) is measured using the TRAK-assay (median (range), in U/l), and the TSH receptor stimulating immunoglobulins (TSI) is measured using a bioassay (cAMP median (range) in pM/well). * P-value for comparison between groups by Mann-Whitney (U), or for Spearman's correlation efficient (significance level α < .05).
Association of thyrotropin receptor antibodies with activity rather than severity of GO

Figure 1. Correlation between TSI and TBII in 63 patients with moderately severe Graves’ ophthalmopathy

Figure 2. Correlation between the Clinical Activity Score (CAS) and TSI (Panel A) and TBII (Panel B) levels in 63 patients with moderately severe Graves’ ophthalmopathy. Spearman’s correlation coefficient, significance level (α < 0.05), and the formula of the line of regression is presented.
Table 2. Eye disease characteristics of 63 patients with moderately severe Graves' ophthalmopathy related to the levels of TBII and TSI.

<table>
<thead>
<tr>
<th></th>
<th>TBII Median (range)</th>
<th>r</th>
<th>p value</th>
<th>TSI Median (range)</th>
<th>r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>duration GO (months, median and range)</td>
<td>15 (4-240)</td>
<td>-0.05</td>
<td>.67</td>
<td></td>
<td>0.07</td>
<td>.59</td>
</tr>
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<td>severity GO</td>
<td></td>
<td></td>
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<td>class 2: 0 or a</td>
<td>18</td>
<td></td>
<td></td>
<td>7 (2,5-400)</td>
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<td></td>
</tr>
<tr>
<td>b or c</td>
<td>45</td>
<td></td>
<td></td>
<td>20 (2,5-274)</td>
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<tr>
<td>class 3: mm (mean±sd)</td>
<td>63</td>
<td></td>
<td>.36</td>
<td>19.9±2,5</td>
<td></td>
<td>.004</td>
</tr>
<tr>
<td>class 4: 0 or a</td>
<td>15</td>
<td></td>
<td></td>
<td>26 (2,5-400)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b or c</td>
<td>48</td>
<td></td>
<td></td>
<td>13 (2,5-21,5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>elevation in° (mean±sd)</td>
<td>18.4±12.3</td>
<td></td>
<td>.13</td>
<td>.35</td>
<td></td>
<td>.52</td>
</tr>
<tr>
<td>Total Eye Score (TES)</td>
<td>12.5±3.3</td>
<td></td>
<td>.13</td>
<td>.33</td>
<td></td>
<td>.37</td>
</tr>
<tr>
<td>Clinical Activity Score (CAS)</td>
<td>3.3±1.8</td>
<td></td>
<td>.54</td>
<td>.0001</td>
<td></td>
<td>.0001</td>
</tr>
</tbody>
</table>

TBII receptor binding inhibiting immunoglobulins is measured using the TRAK-assay (median (range), in U/l), and the TSH receptor stimulating immunoglobulins (TSI) is measured using a bioassay (cAMP median (range) in pM/well). P-value for comparison between groups by Mann-Whitney (U); or for Spearman's correlation coefficient (significance level α < .05).
Discussion

There is increasing evidence that the TSH-R is present in orbital tissue. TSH-R mRNA has been found in tissue homogenates by PCR,\textsuperscript{17} and immunocytochemistry has made it likely that orbital fibroblasts express the receptor at certain stages of their maturation.\textsuperscript{18} These findings support an old hypothesis that TSH-R antibodies might not only cause Graves' hyperthyroidism, but are also responsible for Graves' ophthalmopathy by cross-reacting with a shared autoantigen.\textsuperscript{19} However, the mere presence of a TSH-R on orbital fibroblasts does not prove that TSH-R autoantibodies indeed cause, or initiate the swelling of orbital tissues. There is limited evidence that TSI or TSH have an effect on orbital fibroblasts in vitro. The studies by Rotella et al.,\textsuperscript{20} showing that TSH increases glycosaminoglycan production by orbital fibroblasts, and Heufelder et al.,\textsuperscript{21} showing upregulation of ICAM-1 on orbital fibroblasts upon incubation with TSH, are the only evidence for this and need confirmation. Thus, whether there is an interaction between TSI and a TSH-R in the orbit still needs to be proven.

Except for these in vitro studies another way to support the hypothesis that TSI play a pathogenetic role in GO would be to show that TSI titers in some way correlate with the clinical expression of the ophthalmopathy. This has been investigated in the past, and most authors could not find such a relation,\textsuperscript{5-9} leading to a consensus in the 1980s that TSI were not involved in GO.\textsuperscript{22} However, many of these studies used insensitive TSI assays (including the LATS bioassay), and sought for a correlation with disease severity using rather crude ways to assess this. Moreover, the approach to relate TSI with disease severity can be seriously challenged. From the studies of Rundle and colleagues we know that GO has a tendency to spontaneously ameliorate over time, usually without complete remission to the pre-morbid state.\textsuperscript{23} Thus, even in this burnt-out, fibrotic, inactive stage the patients might still have considerable proptosis and suffer from diplopia. However, by this time the Graves' disease might have become inactive with low
TSI levels. In other words, in inactive eye disease a possibly pathogenetic factor, like TSI, will not correlate any more with eye disease severity, a bias that was not recognized in the previously mentioned studies.

We, therefore, used a different approach and correlated TBII and TSI levels with the CAS, a parameter for eye disease activity, and found a highly significant correlation between CAS and both TBII and TSI values. This observation thus lends clinical support for TSI being important in the pathogenesis of GO. It is further supported by the significant correlations with severity of soft-tissue involvement and proptosis, though not with an overall parameter of disease severity (TES).

Which are the limitations of our study? First, we only found an association, which of course does not prove causation. Secondly, the coexisting thyroid disease might be a more prominent determinant of TSI levels. However, all our patients were rendered euthyroid for at least 2 months mostly by antithyroid drugs, and none had received radioactive iodine in the previous six months. Also, TBII and TSI levels did not correlate with the duration of the thyroid disease, nor with another parameter of thyroid autoimmunity, e.g. TPO antibody levels. Thirdly, TSI measurements are known to have a high intra-assay variability. However, we measured all TSI samples in one assay run, and the correlation with the CAS was also found with the less sensitive TBII assay.

In conclusion, our study establishes a significant correlation between TBII or TSI titers and the clinical activity of Graves' ophthalmopathy. These results are compatible with a role of these TSH-R antibodies in the pathogenesis of thyroid associated ophthalmopathy.

References


**Addendum**

**TSH-RECEPTOR AUTOANTIBODIES AS PREDICTORS OF OUTCOME OF IMMUNOSUPPRESSION IN GRAVES' OPHTHALMOPATHY.**

In view of the observed relationship between TSI and TBII levels with not only the severity of the eye disease but also -and even to a larger extent- with the activity of the eye disease, we established if TSH receptor antibodies might have predictive value for the outcome of immunosuppression. This was investigated in the same 63 patients with moderately severe Graves' ophthalmopathy, who all were treated with retrobulbar irradiation. The therapeutic outcome was assessed after 6 months.

34 patients were responders and 29 nonresponders (no change in 24 patients, and deterioration in 5 patients). At baseline there was no significant difference in TBII or TSI levels between both groups (Table 1). The ROC curves for TBII and TSI had a disappointingly low area under the curve (0.59 ± 0.07 for TBII, 0.53 ± 0.08 for TSI, see figure 1).

We also followed the course of serum TBII and TSI levels in the six months after radiotherapy. We found a significant decrease in titers of both TBII and TSI, which was similar in responders and nonresponders (Wilcoxon's matched pairs, p < 0.0001 for n=58 pairs) (table 1 and figure 2).

It is concluded that despite the correlation of TBII and TSI levels with the CAS, the TSH receptor antibodies in serum do not reliably predict the outcome of radiotherapy. What might be the explanation for this? First, the CAS itself performed rather poorly in predicting a response in this cohort (CAS > 4/10 had a positive predictive value of 65%, and a negative predictive value of 56% in
predicting a response to immunosuppression). Secondly, TBII and TSI titers also correlate with the severity of the ophthalmopathy. Thirdly, it might be that the titers are also determined by the thyroid disease itself, which was treated by antithyroid drugs. This might explain why the titers decreased during follow up in all patients.

One might speculate that TSH-R antibodies are involved in the initiation of the autoimmune response in the orbit by cross-reacting with an orbital TSH-R.\(^1\) The perpetuation of the orbital autoimmune process might very well be determined by a plethora of secondary events, including cytokine release by infiltrating lymphocytes, or the induction of the formation of secondary autoantibodies to orbital (not thyroidal) antigens. On the other hand, a high TBII or TSI level may simply reflect a more severe autoimmune dysregulation associated with more severe manifestations of Graves’ disease in the thyroid, and outside the thyroid in the orbit, and even the pretibial skin.

Table 1. Serum concentrations of TSH receptor antibodies, TSH, FT4 and T3 in 63 patients with moderately severe Graves’ ophthalmopathy, measured before and six months after retrobulbar irradiation.

<table>
<thead>
<tr>
<th></th>
<th>t=0</th>
<th>t=0</th>
<th>t=0</th>
<th>p*</th>
<th>t=26 weeks</th>
<th>p**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>responders</td>
<td>nonresponders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=63</td>
<td>n=34</td>
<td>n=29</td>
<td></td>
<td>n=63</td>
<td></td>
</tr>
<tr>
<td>TSI (cAMP pM/well)</td>
<td>median (range)</td>
<td>39 (21-130)</td>
<td>39 (23-130)</td>
<td>39 (21-130)</td>
<td>.70</td>
<td>29 (13-130)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBII (U/l)</td>
<td>median (range)</td>
<td>13 (2,5-400)</td>
<td>13 (2,5-274)</td>
<td>13 (2,5-400)</td>
<td>.23</td>
<td>6 (2,5-220)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH</td>
<td>mean (sem)</td>
<td>1,96 (0,28)</td>
<td>1,69 (0,32)</td>
<td>2,27 (0,47)</td>
<td>.44</td>
<td>1,47 (0,23)</td>
</tr>
<tr>
<td>FT4</td>
<td>mean (sem)</td>
<td>16,2 (0,47)</td>
<td>16,5 (0,76)</td>
<td>15,8 (0,56)</td>
<td>.50</td>
<td>16,1 (0,47)</td>
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<tr>
<td>Total T3</td>
<td>mean (sem)</td>
<td>1,93 (0,06)</td>
<td>1,93 (0,09)</td>
<td>1,92 (0,09)</td>
<td>.97</td>
<td>1,90 (0,06)</td>
</tr>
</tbody>
</table>

\(P^*, \text{p-value for difference between responders and nonresponders using the Mann-Whitney (U) test; } P^{**}, \text{p-value for difference between } t=0 \text{ and } t=26 \text{ weeks using Wilcoxon’s signed rank test.}\)
Association of thyrotropin receptor antibodies with activity rather than severity of GO

Figure 1. Receiver-operating-characteristic (ROC) -curves of serum TBII (left panel) and TSI (right panel) concentrations for predicting a response to radiotherapy.

Figure 2. Serum TBII (left panel) and TSI (right panel) concentrations at baseline and 26 weeks after radiotherapy in patients with moderately severe Graves' ophthalmopathy.
References

